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Original Article

Unsuccessful effort to detect human papillomavirus DNA in urinary bladder cancers by the polymeras chain reaction and *in situ* hybridization

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The association of human papillomavirus (HPV) with urinary bladder carcinogenesis is now a controversial issue. In order to certify the presence of HPV DNA in urinary bladder cancers, the polymerase chain reaction (PCR) using five primer sets for detecting various HPV types was used in this study as well as *in situ* hybridization (ISH) for HPV 16 and 18 detection. In the PCR study of 93 DNA samples extracted from formalin-fixed and paraffin-embedded urinary bladder cancers, no HPV DNA was detected in these tumor samples. The ISH study was also performed on the same tumor samples, but failed to demonstrate any HPV 16- or 18-positive signals in all except one of the tumor samples. However, the PCR failed to demonstrate HPV 16 DNA even in the bladder cancer positive for HPV 16 DNA by the ISH. This ISH technique was able to demonstrate HPV 16 and 18 DNA in eight of 13 paraffin-embedded cervical cancers, in which HPV 16 or 18 DNA had already been detected by the PCR. Our HPV study using PCR and ISH revealed that the HPV status of urinary bladder carcinomas was far different from that of cervical cancers.

Key words: bladder cancer, human papillomavirus, *in situ* hybridization, polymerase chain reaction

Human papillomavirus (HPV) is a small DNA virus belonging to the Papovaviridae. In humans, HPV infection is commonly seen in the surface epithelium of the body and usually causes hyperplastic epithelial lesions of the skin and uterine cervix. Recently it has been shown that HPV has oncogenic potential and causes premalignant and malignant lesions in several organs, especially in the uterine cervix.[1] Now, various HPV DNA can be demonstrated in more than 90% of premalignant and malignant lesions of the uterine cervix and are suspected of playing an important role in cervical carcinogenesis.[1] Recent advances in tumor biology have elucidated the precise oncogenic mechanism of HPV. HPV gene products E6 and E7 proteins bind to cellular tumor suppresser gene products Rb and p53 proteins, which results in their losing their ability to regulate the cell cycle.[2] These findings show that abnormality of the tumor suppresser gene is crucial for cervical carcinogenesis. In fact, an inverse relation betw en p53 abnormality and HPV infection has already been demonstrated in cervical cancers.[3]

Up to now more than 70 subtypes of HPV have been isolated from various lesions.[4] Although about 15 HPV subtypes are detected in various premalignant and malignant lesions of the uterine cervix, less than 10 of them are found only in the malignant lesions of the cervix.[1,4] Among them, HPV 16 and 18 are the most common viral subtypes found in these malignant lesions and are regarded as HPV subtyp s with strong malignant potential in the cervical epithelium. Therefore, the identification of HPV subtypes in various cervical lesions is clinically useful for predicting the patient's outcome.[1]

Recently HPV DNA has been demonstrated in various cancer tissues of the urinary bladder, prostate, aerodigestive tract, including the lung, larynx and esophagus, and the sebaceous gland in addition to anogenital and cervical cancers.[1,5] However, it has not been concluded that HPV plays a significant role in carcinogenesis in these organs. Of these organs, the anatomical close proximity of the urinary bladder mucosa to the anogenital area, particularly in females, has led to the suggestion that HPV may be involved in urinary bladder carcinogenesis. In this study, we tried to confirm the presence of HPV DNA in urinary bladder cancers and to determine the role of HPV infection in urinary bladder carcinogenesis.

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MATERIALS AND METHODS

Tumor specimens

Bladder carcinoma cas s were collected from the pathology files of the Gunma University and its affiliated hospitals during the period from 1989 to 1993. A total of 93 tumor specimens obtained at biopsy or surgery from 93 bladder cancer patients (63 males and 30 females) were used in the study. All tumor specimens had been fixed in 15% formalin and embedded in paraffin for routine pathological diagnosis. Pathological diagnosis and histological grading were reassessed according to the criteria of *The General Rule for Clinical and Pathological Studies on Bladder Cancer of the Japanese Urological Association and the Japanese Society of Pathology*. The tumor specimens included 80 transitional cell carcinomas (grade 1, 9; grade 2, 48; grade 3, 23), 11 squamous cell carcinomas, 1 adenocarcinoma and 1 undifferentiated carcinoma. The histopathological features of the tumor specimens are summarized in Table 1. According to the medical records, no patients suffered from condylomatous lesions in the external urogenital region or from immunosuppression.

All tumor specimens were examined for the presence of HPV DNA by the polymerase chain reaction (PCR) technique and *in situ* hybridization (ISH) under conditions described later. In addition to these bladder cancers, an ISH study was also carried out on 13 cervical cancers, which had been shown to contain HPV DNA by the PCR method (10 tumors for HPV 16 and 3 for HPV 18).

DNA extraction

A paraffin block of specimen showing representative histology of bladder cancer was selected and used for tumor DNA extraction. Several 6 μm thick sections were cut from each block by a microtome with a new disposable blade and collected in a 1.5 mL tube. The sections were digested in 0.5 mL of a buffer containing 50 mmol/L Tris-HCl (pH 8.5), 1 mmol/L EDTA, 200 μg of proteinase K/mL, and 0.5% Tween-20 for 4–8 h at 62°C. After the addition of 30% Chelex-100 (Bio-Rad, Richmond, CA, USA), the samples were boiled for 10 min then centrifuged at 10 000 rpm for 5 min at 4°C.[6] The liquid phase under the paraffin layer was used for PCR amplification.

PCR for HPV-DNA detection

The HPV target sequence was amplified in a 50 μL reaction mixture consisting of 5 μL of the DNA sample, 20 mmol/L Tris-HCl (pH 8.2), 10 mmol/L KCl, 6 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgCl_2$, 1% Triton X-100, 100 ng of nuclease-free BSA per mL, 100 μmol/L each of dNTP, 1 μmol/L each of oligonucleotide primers and 1 U of *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The two type-specific primers for HPV 16 and 18 and three sets of consensus primers for a wide variety of HPV subtypes were used to amplify different open reading frames of HPV genomes.[7–9] The information about the primers used in this study is summarized in Table 2. In addition, a pair of primers to amplify the 162 bp of the c-Ki-*ras* 2 gene including codons 1 to 36 was used to check the validity of the template DNA.[10]

The mixture was denatured initially for 1 min at 98°C, followed by 40 cycles of amplification using a GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT, USA). In the case of amplification with the type specific primers and the consensus primers pU-1M/pU-2R and pU-31B/pU-2R, each cycle consisted of denaturing at 98°C for 15 s, annealing at 55°C for 30 s, and extending at 74°C for 1 min. In the case of amplification with consensus primers L1C1/L1C2, the annealing was changed to 48°C for 30 s. All PCR amplification reactions were terminated with a single 10 min cycle at 74°C. Finally, the PCR product (10 μL) was electrophoresed

Table 1 Detection of HPV DNA by PCR and ISH in urinary bladder cancer

Samples	No. cases	PCR	ISH	
		Positive cases	Positive for HPV 16	Positive for HPV 18
Urinary bladder cancers	93	0	1	0
Transitional cell carcinomas	80	0	1	0
Squamous cell carcinomas	11	0	0	0
Adenocarcinoma	1	0	0	0
Undifferentiated carcinoma	1	0	0	0
Cervical cancers positive for				
HPV 16	10	10(100%)	6(60%)	—
HPV 18	3	3(100%)	—	2(67%)

PCR, polymerase chain reaction; ISH, *in situ* hybridization.

Table 2 Primers used

Type and name of the primer (sense/antisense)	Region	Localization	Length of PCR product	Detectable HPV subtype(s)	Reference
Specific (H1/H2)	E6	320–429	110 bp	16	7
Specific (H1/H3)	E6	328–437	110 bp	18	7
Consensus (pU-31B/pU-2R)	E6–7	400–627	228 bp	6, 11	8
Consensus (pU-1M/pU-2R)	E6–7	(418–693)*	231–268 bp	16, 18, 31, 33, 52b, 58 and more	8
Consensus (L1C1/L1C2)	L1	(5500–6000)*	244–256 bp	6, 11, 16, 18, 31, 33, 42, 52, 58 and more	9

*Approximate base position.
bp, base pairs.

on 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME, USA), and visualized with ultraviolet light after ethidium bromide staining.

For positive controls, purified HPV plasmid DNA and cellular DNA purified from cervical cancers that were known to harbor HPV 6, 11, 16, 18 and 52 were run simultaneously with the test specimens. For negative controls, cellular DNA from a lymph node and distilled water were used instead of template DNA.

To minimize potential contamination, the PCR and analysis of its product were carried out in a laboratory physically separated from the one in which the DNA was isolated from clinical specimens. Also, general anticontamination steps included dedicating separate sets of supplies and pipetting devices, autoclaving buffers and using disposable plastic ware; gloves and face masks.[11]

In situ hybridization

In situ hybridization was performed with fluorescein-5-isothiocyanate (FITC)-labeled probes specific for HPV 16 and 18 (DAKO Japan, Kyoto, Japan). An ISH detection system (DAKO) was used for the detection of FITC-labeled probes as described in the instruction manual. Briefly, 3 μm thick sections cut from the same paraffin block used in the PCR study were first deparaffinized with xylene and then digested with 0.2 N HCl solution containing 0.8% pepsin for 10 min at 37°C. This was followed by simultaneous denaturation of the cellular DNA and the FITC-labeled HPV probe for 6 min at 95°C, and the hybridization was carried out for 2 h at 37°C. After hybridization, the slides were washed under high stringency conditions with 0.1 × standard sodium citrate at 58°C for 30 min. After two washings, the sections were treated with rabbit anti-FITC polyclonal antibody conjugated with alkaline phosphatase for 20 min in a humidified chamber. Finally the sections were colored with nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyphosphate substrate for 2–3 h in the dark at room temperature. The sections were counterstained with methyl green.

RESULTS

The results of this study are summarized in Table 1. The PCR studies using five sets of primers completely failed to detect HPV DNA in any of the DNA samples of the 93 urinary bladder carcinomas. However, these PCR systems clearly demonstrated HPV 16 and 18 DNA in 10 and three of 13 cervical cancers, respectively, which were used as positive controls. During PCR studies for HPV DNA in bladder carcinoma samples, these positive control samples always showed a strong band, as expected from their size. The sensitivity of these PCR systems was evaluated by serial dilution of HPV 16 and 18 plasmid DNA. With each set of primers, the PCR could detect at least 10 fg of HPV plasmid DNA per sample, which corresponded to 0.1 copy/cell (data not shown). In addition to these PCR systems, the 162-bp DNA fragment of the c-Ki-*ras* 2 gene could be amplified in all DNA samples used in this study. Therefore, all extracted tumor DNA samples were considered to be suitable for the PCR reaction as a template.

Using the ISH system for HPV 16 and 18, HPV-positive signals were detected in 6 of the 10 HPV 16-positive cervical cancers and in two of the three HPV 18-positive tumors, respectively. HPV-positive signals were small dots or diffuse fine granules and were exclusively confined to the cancer cell nuclei (Fig. 1a,b). Both dots and diffuse signals in the nuclei were seen in only one HPV 16-positive tumor. In this ISH system, no cross-hybridization between HPV 16 and 18 DNA was observed.

The urinary bladder cancer samples were then examined by ISH but it failed to detect any HPV 16- or 18-positive signals in all except one of these cancers. In that one exception transitional cell carcinoma (TCC, grade 2), HPV 16-positive signals were seen as a fine diffuse pattern of varied intensity and sometimes as ring-shaped on the nuclei of a small number of carcinoma cells (Fig. 1c). These HPV-positive signals were somewhat different from those seen in cervical cancers. As stated before, this cancer also did not yield any positive bands suggestive of the presence of HPV in repeated tests by PCR (Fig. 2).

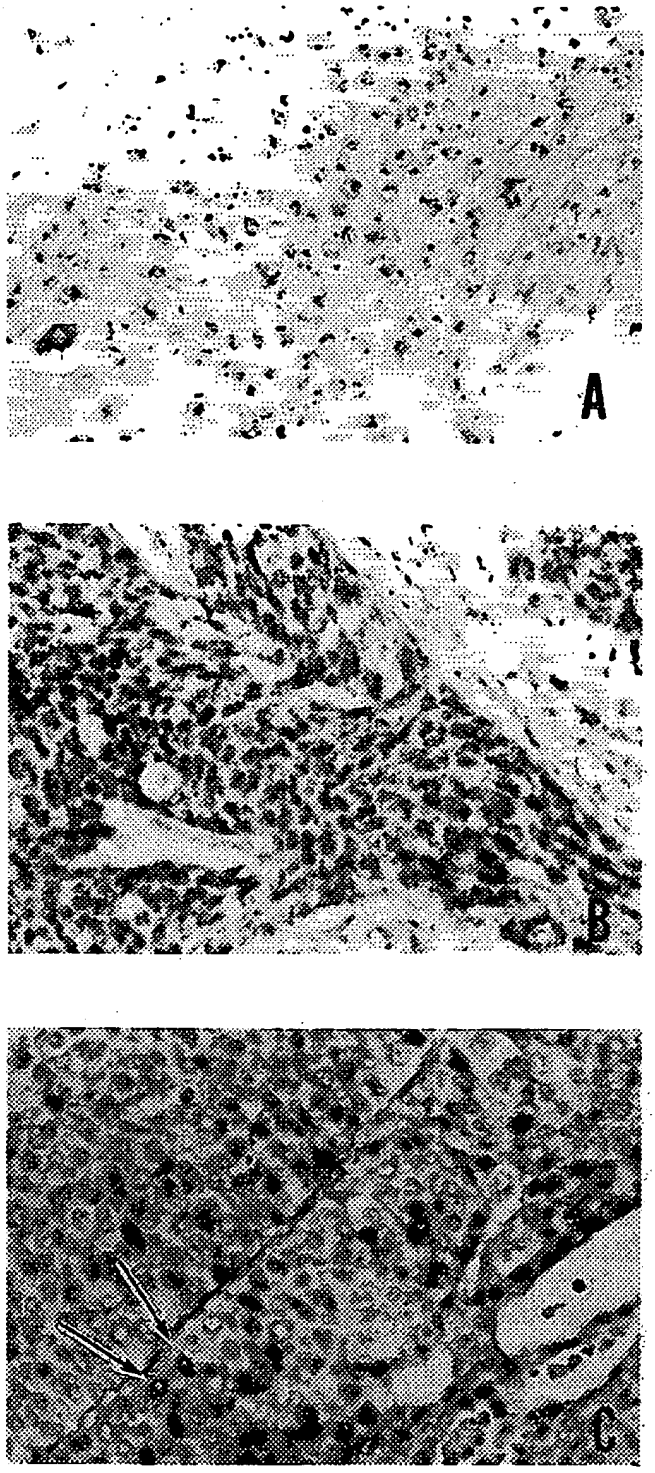

Figure 1 Staining pattern of ISH for HPV 16 DNA. In cervical cancer tissues used as positive controls, positive signals for HPV 16 DNA appear as discrete dots (**A, B**) and a diffuse pattern (**B**) in the nuclei of cancer cells. In a case of TCC, cancer cell nuclei are diffusely stained by ISH (**C**). In addition, several nuclei of cancer cells show a ring-shaped staining pattern (arrows).

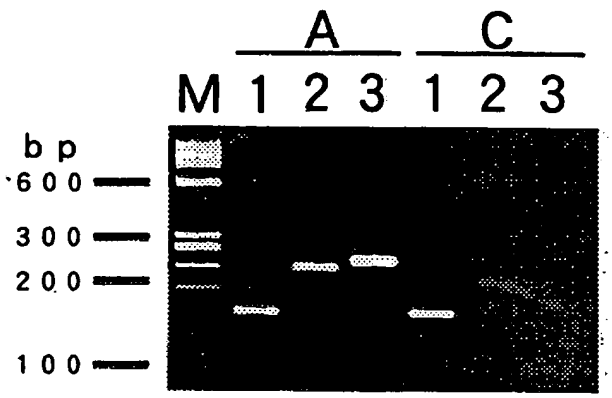

Figure 2 PCR analysis of a TCC positively stained for HPV 16 DNA by ISH. PCR analysis failed to demonstrate HPV 16 DNA in a TCC (C). Cervical cancer DNA is used for a positive control (A). (A) and (C) correspond to the same cases as shown in Fig. 1. Lane M, Marker DNA (Hae III - digested ø X174 DNA); lane 1, PCR product of c-Ki-*ras*; lane 2, PCR product with pU-1M/pU-2R primers; lane 3, PCR product with L1C1/L1C2 primers.

Furthermore, none of the HPV-positive signals was detected in stromal cells, inflammatory cells or normal and dysplastic urinary bladder epithelium adjacent to the tumor.

DISCUSSION

In 1988, Kitamura *et al.* first detected HPV 16 DNA by Southern blot hybridization (SBH) in TCC *in situ* that developed in the urinary bladder of a mildly immunocompromized female patient.[12] Previously she had suffered from several HPV-related diseases such as genital condyloma acuminatum, Bowen's disease of the vulva and cervical carcinoma *in situ*. Since Kitamura *et al.*'s report, at least 14 studies, including the present one, have been carried out to detect the presence of HPV DNA in urinary bladder cancers and are summarized in Table 3.[12-24]

As seen in Table 3, HPV DNA has been searched for in urinary bladder carcinomas by various molecular techniques including SBH, PCR and ISH, and the incidence of HPV in urinary bladder carcinoma has been found to vary from 0 to 81%. However, the majority of these studies revealed that the presence of HPV is uncommon or exceptional in most urinary bladder cancers. On the other hand, at least four studies clearly demonstrated that HPV DNA is frequently present in carcinoma tissues of the urinary bladder. In order to resolve the discrepancies in these HPV studies of urinary bladder cancers, therefore, w tri d to confirm the presence of HPV DNA by the PCR with multiple sets of primers and by ISH for

Table 3 Reports of HPV study in urinary bladder carcinomas

Referenc	No. patients	Method(s) PCR/Target regions	Others	No. positive cases/ total cases (%)	HPV subtype(s) found in positive cases (case no.)
Kitamura (1988)[12]	10	—	SBH	1/10 (10%)	HPV 16*
Bryant (1991)[13]	100	—	ISH	12/100 (12%)	HPV 16/18 (12)
Kerley (1991)[14]	22	PCR/?	ISH	1/22 (4.5%)	HPV 11†
Shibutani (1992)[15]	23	—	SBH	6/23 (26%)	HPV 6/11 (3), HPV 16/18 (2), HPV 31/33 (1)
Chetsanga (1992)[16]	44	PCR/L1	—	1/44 (2.2%)	HPV 16
Knowles (1992)[17]	120	PCR/E1 and L1	SBH	0/120 (0%)	—
Anwar (1992)[18]	48	PCR/E6	ISH	39‡/48 (81%)	HPV 6/11 (22), HPV 16 (13), HPV 18 (18), HPV 33 (14)
Saltzstein (1993)[19]	33	PCR/E1 and L1	—	0/33 (0%)	—
Wilczynski (1993)[20]	22	PCR/E6, E7 and L1	SBH	1/22 (4.5%)	HPV 6†
Sinclair (1993)[21]	14	PCR/L1	.....	0/14 (0%)	—
Furihata (1993)[22]	90	—	ISH	28‡/90 (31%)	HPV 16 (19), HPV 18 (17), HPV 33 (16)
Maloney (1994)[23]	42	PCR/E6 and L1	—	1/42 (2.3%)	HPV 18*†
Chang (1994)[24]	108	PCR/L1	ISH	0/108 (0%)	—
Present study	93	PCR/E6, E6–7 and L1	ISH	0/93 (0%)	—

*Immunosuppressed patients.
†These cases were SCC of the urinary bladder.
‡Double or multiple HPV types were detected in these cases.
PCR, polymerase chain reaction; ISH, *in situ* hybridization; SBH, Southern blot hybridization.

HPV 16 and 18. No HPV DNA was detected by these techniques, which corroborates the results of most HPV studies on urinary bladder carcinomas and the findings are completely identical to those reported by Knowles,[17] Saltzstein *et al*,[19] Sinclair *et al.*[21] and very recently by Chang *et al.*[24]

The failure to detect any HPV DNA in the present study is due to either false- or true-negative results for viral DNA. Theoretically, false-negative findings by PCR might result from: (i) the presence of a substance inhibitory to DNA polymerase;[25] (ii) the limits of the ability to detect HPV; and (iii) unknown HPV subtypes out of the range of detection in this PCR system. However, the possibility of the presence of an inhibitor is negligible because of the successful amplification of the c-Ki-*ras* 2 gene by the PCR. This study used multiple sets of primers to amplify various subgenomic fragments including the E6, E7 and L1 regions of various HPV subtypes to increase the sensitivity and to preclude a negative result caused by the loss of a HPV subgenomic region.[1,26] It is still possible that urinary bladder carcinomas contain a small number of HPV beyond the sensitivity of this PCR system, or a novel unknown HPV subtype, but this speculation is probably unreasonable in view of other HPV studies of cervical cancers using this PCR system.[8,9]

In several studies, HPV DNA was detected in a few cases of urinary bladder carcinoma by various methods including PCR. Chetsanga *et al.* found HPV 16 in only one of 44 TCC by PCR.[16] And three other groups[14,20,23] detected HPV 6, 11 and 18, respectively, in one case each of squamous cell carcinoma (SCC) of the urinary bladder (Table 3), of which one occurred in an immunosuppressed patient similar to the first one reported by Kitamura *et al.*[12] In HPV carcinogenesis in human skin, it is a well-known fact that malignant transformation is closely associated with immunodeficiency.[1]

In humans, HPV has a specific tropism for squamous epithelium and usually causes a hyperplastic epithelial lesion called condyloma. Several well-documented cases of condyloma acuminatum arising in the urinary bladder have been reported to contain HPV 6/11, like anogenital condylomata.[27,28] It seems likely that HPV infection is involved in the development of SCC through the progression of benign condylomata in the urinary bladder. However, Maloney *et al.* reported only one of 22 SCC patients was positive for HPV 18 and this patient was in an immunosuppressive state.[23] Also, the present study failed to demonstrate HPV DNA in 11 cases of SCC of the urinary bladder. From these data, the role of HPV in the development of urinary bladder SCC remains obscure and further investigations are necessary to draw any conclusion. Supposing that HPV infection is related to the tumorigenesis of SCC in the urinary bladder, however, the role of HPV in carcinogenesis in the urinary bladder would be limited because SCC accounts for less than 5% of urinary bladder carcinomas and often occurs in some particular circumstances involving severe inflammation, such as schistosomiasis.[29]

By contrast, a few reports strongly supported a significant association of HPV DNA with urinary bladder carcinomas

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including TCC. Among them, Anwar *et al.* reported that 81% of urinary bladder carcinomas were positive for HPV DNA by PCR with type-specific primers amplifying the E6 region.[18] In their study, a high incidence of HPV 18 and 6/11 and multiple infection with HPV types 6/11, 16, 18 and 33 were found to be characteristic features of urinary bladder cancers. In contrast with the large number of HPV studies on cervical cancers, [8,9,30] their results are unique. In addition, the occurrence of HPV in their studies is so high compared to other studies on bladder cancer that it is possible that HPV DNA might have contaminated the PCR system. Interestingly, Sinclair *et al.* stated that when the PCR was conducted at too low an annealing temperature, a positive band for HPV appeared in all samples of bladder cancers and the negative DNA controls.[21]

The ISH technique has been used to detect HPV DNA in urinary bladder carcinomas as well as in cervical cancers. Because of recent advances in non-isotopic ISH it is now possible to determine the physical state of HPV, either integrated or episomal, in preneoplastic and neoplastic cervical lesions.[31] The episomal HPV shows diffuse signals over the entire nucleus and the integrated form of HPV DNA is seen as small punctuated spots. These staining patterns were also obtained in the positive control samples of cervical cancer in our ISH. Furihata *et al.* detected several HPV DNA in 31% of urinary bladder carcinomas by non-isotopic ISH, but they never stated the physical state of HPV DNA.[22] However, Chang *et al.* very recently confirmed the absence of any HPV DNA in urinary bladder carcinomas by both non-isotopic ISH and PCR techniques,[24] of which the results are identical to ours. Among various molecular techniques to detect HPV DNA, the ISH technique is much inferior in sensitivity and specificity. Also in this current study, PCR failed to detect any HPV DNA in a TCC in which ISH staining suggested HPV DNA. We considered that the ISH signal of that sample was not significant for HPV presence and was a non-specific reaction. In general, ISH-positive findings should be confirmed by other sophisticated molecular techniques such as SBH or PCR. Without those techniques, an overestimation in the interpretation of ISH cannot be excluded.[32]

In summary, we failed to detect any HPV DNA in urinary bladder carcinomas including SCC by both ISH and PCR techniques. These results indicate that HPV infection might not play a significant role in carcinogenesis in the urinary bladder.

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